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Pattern of Deletions of the Dystrophin Gene in Mexican Duchenne/Becker Muscular Dystropy Patients: The Use of New Designed Primers for the Analysis of the Major Deletion "Hot Spot" Region

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We have analyzed 59 unrelated Mexican Duchenne/Becker muscular dystrophy patients (DMD/BMD) using PCR analysis of the 2 prone deletion regions in the DMD gene. Thirty one (52%) of the patients had a deletion of one or several of the exons. Most of the alterations (87%) were clustered in exons 44-52, this being the highest percentage reported until now. In order to improve the molecular diagnosis in the Mexican population, we designed a new multiplex assay to PCR amplify exons 44-52. This assay allowed for the identification of a greater number of deletions in this region compared with the 9 and 5-plex assays previously described and to determine most of the deletion end boundaries. This is a reliable alternative for the initial screening of the DMD patients in the Mexican population. Am. J. Med. Genet. 70:240–246, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: Duchenne muscular dystrophy gene; Mexican patients; deletions distribution; new designed primers; PCR amplification

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked muscular disorder with a prevalence of 1 in 3500 live-born males. DMD is allelic to the milder and less frequent Becker muscular dystrophy (BMD). These hereditary diseases are caused by defects in the dystrophin gene, which consists of 79 exons [Roberts et al., 1993] distributed in approximately 2.5 megabases and encodes a 14 Kb messenger RNA [Koenig et al., 1987] . The protein product of the gene, dystrophin, with a molecular weight of 427 kDa, has been identified as a cytoskeletal membrane protein in striated muscle cells [Arahata et al., 1988; Koenig et al., 1988; Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988].

Analysis of the DNA of DMD/BMD patients have shown that in 50-70% of the cases these phenotypes are the result of deletions mainly localized to either the proximal or central portion of the dystrophin gene, minor and major "hot spots" of deletions, respectively [Darras et al., 1988; Baumbach et al., 1989; Den Dunnen et al., 1989; Gillard et al., 1989; Lindlof et al., 1989]. Partial gene duplications have been reported in 10% of patients. In approximately one third of the cases where no large DNA rearrangements can be detected, point-mutations and small insertions/deletions may be responsible for the deficiency [Reninsland and Reiss, 1994] .

The detection of deletions in the dystrophin gene was performed by Southern blot analysis using the cDNA as DNA probes. However, the polymerase chain reaction (PCR) was applied more recently for a rapid and simple detection of deletions in DMD and BMD patients [Beggs et al., 1990; Chamberlain et al., 1990]. This nonisotopic method, based on the simultaneous

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amplification of several deletion-prone exons within the dystrophin gene, detects the vast majority of dystrophin gene deletions.

While many studies have been carried out in North American, European and Asian populations, there are few reports on Latin American populations [Passos-Bueno et al., 1990; Passos-Bueno et al., 1992; Coral-Vazquez et al., 1993]. In this study, the DNA of 59 DMD/BMD unrelated Mexican patients was analyzed by multiplex amplification of 16 exons located in the deletion-prone regions of the dystrophin gene. Almost all the detected deletions (87%) were located in the central portion of the dystrophin gene (exons 44-52). We designed a new set of primers, which detect mutations located at the major deletion "hot spot" and determined the exon boundaries of most of these. The frequency and distribution of the dystrophin gene deletions found in Mexican patients were compared with those present in other populations.

METHODS

Fifty nine DMD/BMD patients from unrelated families enrolled at the "Hospital Infantil de México, Dr. Federico Gómez", "Hospiral de Pediatria, Centro Médico Nacional Siglo XXI-IMSS", "Hospital General-SSA" and "Instituto Nacional de Ortopedia-SSA" were studied. Patients were diagnosed by standard clinical and laboratory criteria for DMD/BMD, such as creatinine kinase (CK) levels and myopathic changes detectable by muscle biopsy and electromyography. All subjects enrolled in this study were born in México. Genomic DNA was extracted from leukocytes as described by Kempter [1992] except that DNA was purified by phenol extraction. Multiplex DNA amplifications, using the 9-plex and 5-plex oligonucleotides described by Chamberlain et al. [1990] and Beggs et al.[1990], were carried out according to these authors. PCR primers were obtained from the Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX, USA. Genomic DNA was also amplified by PCR using 3 sets of 3-plex reactions for the exons 44/48/49; 45/46/52 and 47/50/51. Primers were designed using the cDNA sequence [Koenig et al., 1987] and the exon structure of the DMD gene [Roberts et al., 1993]. Sequences of the primers are as follows: DMD-44F, 5′-CGATTTGACAGATCTGTTGAG; DMD-44R, GATACCATTTGTATTTAGCATGTTCC; DMD-45F, 5′-GAACTCCAGGATGGCATTGG; DMD-45R, 5′-CTGTCTGACAGCTGTTTGCAG; DMD-46F, 5′-GCTAGAAGAACAAAAGAATA TCTTG; DMD-46R, 5′-CTTGACTTGCTCAAGCTTTTC; DMD-47F, 5′-TGGTGGAAGAGTTGCCCCTG; DMD-47R, CTTTTATCCACTGGAGATTTGTCTG; DMD-48F, 5′-GTTTCCAGAGCTTTACCTGAG; DMD-48R, 5′-CTGAACGTCAAATGGTCCTTCTTG; DMD-49F, 5′-GAAAACTGAAATAGCAGTTCAAGC; DMD-49R, 5′-CTTCACTGGCTGAGTGGCTG; DMD-50F, 5′-GGAAGTTAGAAGATCTGAGCTC; DMD-50R, 5′-GGCTCCAATAGTGGTCAGTC; DMD-51F, 5′-CTCCTACTCAGACTGTTACTCTG; DMD-51R, 5′-CTTCTGCTTGATGATCATCTCG; DMD-52F, GCAACAATGCAGGATTTGGAAC; DMD-52R, 5′-CGATCCGTAATGATTGTTCTAGC. Standard PCR conditions were as follows: reaction mixtures consisted of 200-400 ng of genomic DNA, 25 pmol of each primer, 200 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3, triton X-100 and 2 units of Taq polymerase (Perkin-Elmer Cetus) in a volume of 100 μl. Amplification was carried out by using a Perkin Elmer Cetus thermal cycler; one cycle 5 min at 94°C, followed by 30 cycles for 1 min at 94°C, 1 min at 55°C, 2 min at 62°C, and a final elongation step of 5 min. The PCR products were analyzed on 6% polyacrylamide electrophoresis gels stained with ethidium bromide. To perform Southern blot analysis, DNA from patients was digested with HindIII restriction enzyme, electrophoretically separated on 0.8% agarose gels and transferred to Hybond-N (Amersham). The membranes were hybridized with ^{32}P-dCTP random-primed dystrophin cDNA clone 9, and autoradiographs were processed at -70°C for 2-7 days.

RESULTS

Fifty-nine DMD and BMD unrelated Mexican patients were classified as non-ambulatory and ambulatory. In the first group, patients were classified as DMD if they were wheelchair bound before age 12 years and BMD if they were still ambulant at age 16. Patients were classified as intermediate if they became wheelchair bound between the ages of 12 and 16 years. In the second group, patients were classified as DMD or BMD according to clinical evolution and family history. Thirty-nine patients presented DMD, 4 had the BMD phenotype, 2 showed an intermediate severity, and 14 were too young to be classified. Of this group 40 were apparent sporadic cases, 12 familial cases and in 7 this information was not available. Molecular analysis of the patient's dystrophin gene was performed by multiplex PCR reactions using the 9-plex [Chamberlain et al., 1990] and 5-plex [Beggs et al., 1990] amplifications (Fig.1A and B respectively). Deletions were found in 21 of the 39 DMD patients (~54%), in 2 of 4 BMD patients (50%), in the 2 intermediate patients (100%) and in 4 among 14 unclassified patients (~29%). Familial and isolated cases had the same percentage of mutations (50%). Three deletions were detected toward the 5' end of the gene, in exons 4, 8 and 17 while 25 were located at the major deletion "hot spot". One patient had a deletion extending at least from the muscle promoter to exon 52, since no amplification products were obtained by the 9-plex and 5-plex assays (Fig. 2A). To determine the exon boundaries of deletions located in the major deletion "hot spot" of the DMD gene, patient's DNA was further screened with 9 pairs of oligonucleotides designed for this study. Primers were chosen to amplify each one of the major "hot spot" exons (44 to 52), excluding any intron fragment. Since all the exons in this region were amplified, it was possible to detect the effect of each of these deletions on the dystrophin translational reading frame. To facilitate resolution of each fragment by agarose gel electrophoresis following amplification reactions, the 9 pairs of primers were clustered in 3 different reactions: exons 45/ 46/52, PCR fragments of 167, 148 and 116 bp, respectively and 47/50/51, PCR fragments of 147, 106 and 232 bp,

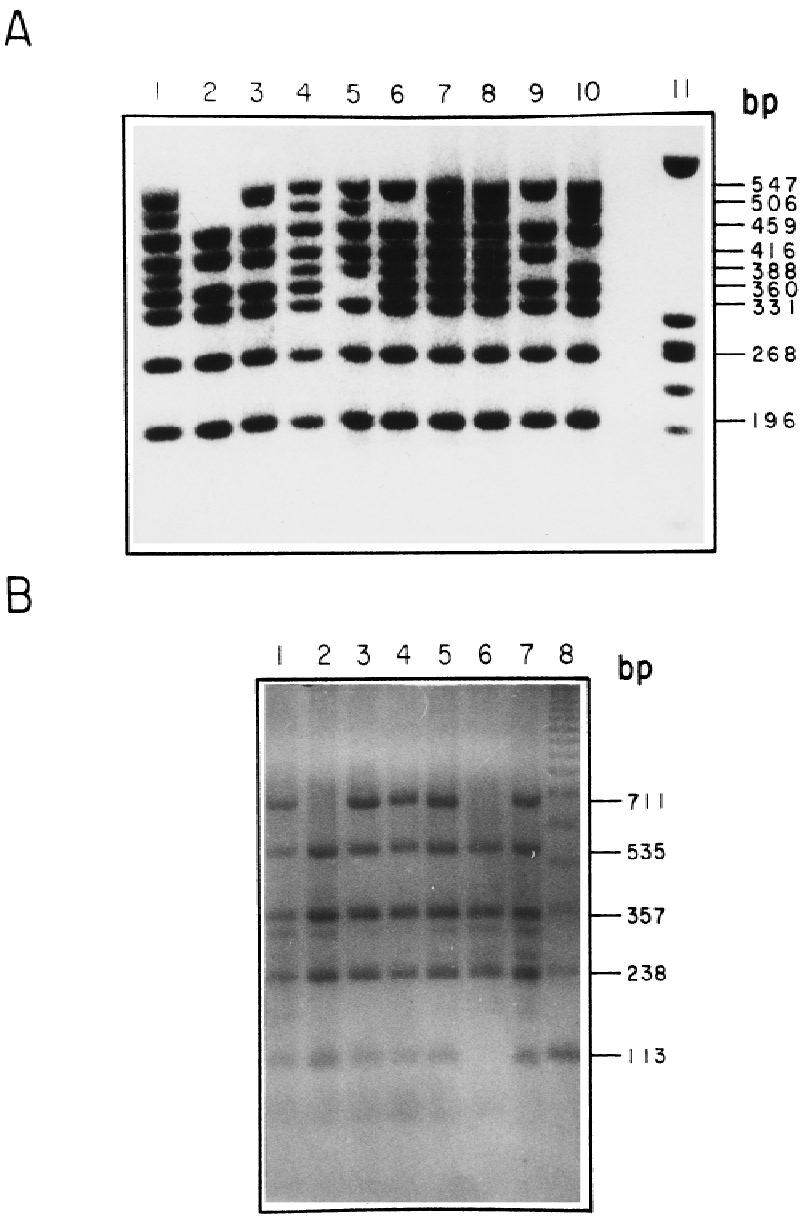

Fig. 1. 9-plex and 5-plex DNA amplifications of genomic DNA from unrelated male DMD/BMD patients. DNA samples were amplified as described in Materials and Methods and PCR products were electrophoresed on 1.4% agarose gels containing ethidium bromide. **A:** Samples analyzed by DNA amplification using the 9-plex oligonucleotides: **lane 1:** control male; **lanes 2-10:** DMD/BMD patients; **lane 11:** DNA marker (HaeIII digest 0174 DNA). Lanes 2, 3, 5, 6, 9 and 10 display deletions of one or several exons. **B:** DNA samples analyzed using the 5-plex oligonucleotides: **lane 1:** DNA from control male; **lanes 2-7:** DMD/BMD patients; **lane 8:** marker DNA (HaeIII-digested 0174 DNA); lanes 2 and 6 display deletions. To the right of A and B, the size of the amplified fragments in base pairs (bp) is indicated.

respectively (Fig. 3A); 44/48/49, PCR fragments of 142, 186 and 102 bp, respectively (Fig. 3B).

Screening with the 3 sets of primers allowed verification of all of the deletions previously detected in the major "hot spot" by the 9-plex and 5-plex assays. Two additional deletions encompassing exons 46-47 were also identified (Fig. 2B). Altogether, 9-plex, 5-plex and the three 3-plex assays detected deletions in 31 (~53%) out of 59 patients. Among 31 patients with deletions, 27 (87%) were confined within exons 44-52 (Fig. 2). Of 27 mutations in the major deletion "hot spot", 9 cases had a deletion in only one exon.

The endpoints of all the deletions encompassing the 44-52 exons were determined by using the three 3-plex assays while mutations with borders farther than exon 52 were analyzed by Southern blot using the cDNA probe 9 [Koenig et al., 1987]. It was evident that the 5′ limits of the deletions were more homogeneous than the 3′limits and the ends were mainly located between exons 44-45 (~30%), 45-46 (~30%), 47-48 (~22%) and 50-51 (~33%) (Fig. 2B).

The effect of 22/27 deletions at the major "hot spot" region over the dystrophin translational reading frame was examined; 13 DMD patients, the two individuals with intermediate severity, 1 BMD patient and 3 unclassified patients had out-of-frame deletions while two

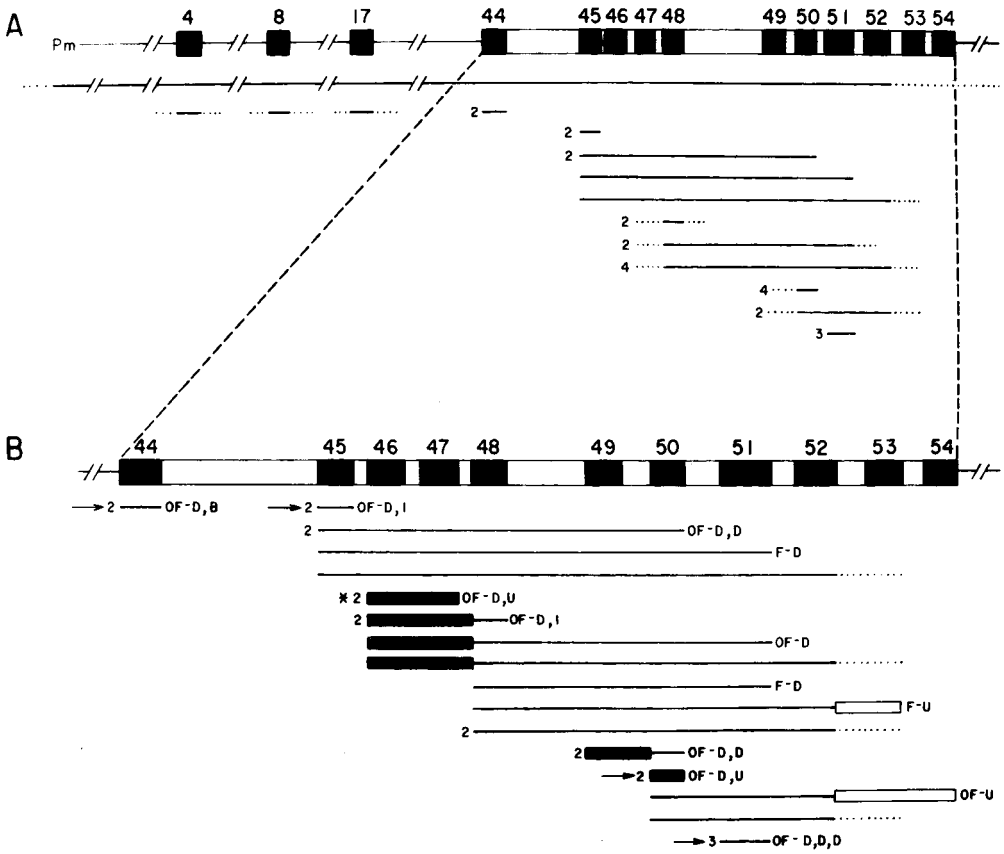

Fig. 2. Distribution of deletions found in 31 DMD/BMD Mexican patients. Results were obtained after the analysis with the 9-plex and 5-plex **(A)** and with the three 3-plex assays **(B).** A partial schematic illustration of the DMD gene with exons (solid boxes) and introns (open boxes) is shown on the top of each panel. Below these are represented the extent of the deletions observed with the 9 and 5-plex assays (lines) and the limits obtained with the three 3-plex assays (solid bars) and Southern blot (open bars) analyses. Dashed lines denote unknown limits. Arrows point at deletions encompassing only one exon. Numbers at the left of deletions indicate the number of independent deletions detected at the same region and an asterisk indicates the deletions that were exclusively detected by the 3-plex assays. D, DMD patients; B, BMD patients; I, intermediate severity; U, unclassified patients. OF, corresponds to out of frame deletions and F to in frame deletions.

DMD patients and one unclassified patient had in-frame deletions (Fig. 2B). In the remaining 5 patients we were not able to determine the deletion boundaries, due to the lack of enough DNA sample.

DISCUSSION

The deletion rate observed in the Mexican DMD/BMD patients (~53%) agrees with the results reported in other populations (Table I) with the exception of the percentages determined for the Israeli [Shomrat et al., 1994] and the black population of South Africa [Ballo et al., 1994] in which the rates were 37% and 22%, respectively. In the Mexican population there are 2 regions of the gene prone to deletions, as noted by others [Forrest et al., 1987; Koenig et al., 1987; Darras et al., 1988; Baumbach et al., 1989]. In our sample, 87% of the detected deletions were located in the major "hot spot", this being the highest percentage reported until now (Table I). However, this percentage may be due to the more extensive analysis done on the central region compared with that of the 5′end. Alternatively, this particular distribution of deletions could be a consequence of the "local DNA sequence environment" which may be relevant in the accumulation of differences in the intron sequences of different populations and ultimately in the origin of deletions and mutations [Cooper and Krawezak, 1991; Danieli et al., 1993; Krawezak and Cooper, 1993; Florentin et al., 1995].

Taking into account the pattern of deletions on the DMD gene in the Mexican population obtained with the 9-plex and 5-plex assays, we designed three different 3-plex assays to detect the deletions encompassing exons 44-52 and determine their exon boundaries. Using the three 3-plex assays the number of deletions detected in the "hot spot" region was increased. Furthermore, by using only the 3-plex for the exons 47/50/51 it was possible to identify 77% of the alterations observed with the 9-plex and 5-plex. These results demonstrate the usefulness of the 3-plex assays for the Mexican DMD/BMD patients analysis.

In this study, deletion breakpoints were located mainly in introns 44 (30%), 45 (30%) and 50 (37%). Several authors have previously shown that intron 44 is the most frequent site for rearrangements in the DMD gene [Den Dunnen et al., 1989; Upadhyaya et al., 1990; Kitoh et al., 1992], whereas in the Greek population, as in our population, a major "hot spot" was located in intron 50 [Florentin et al., 1995].

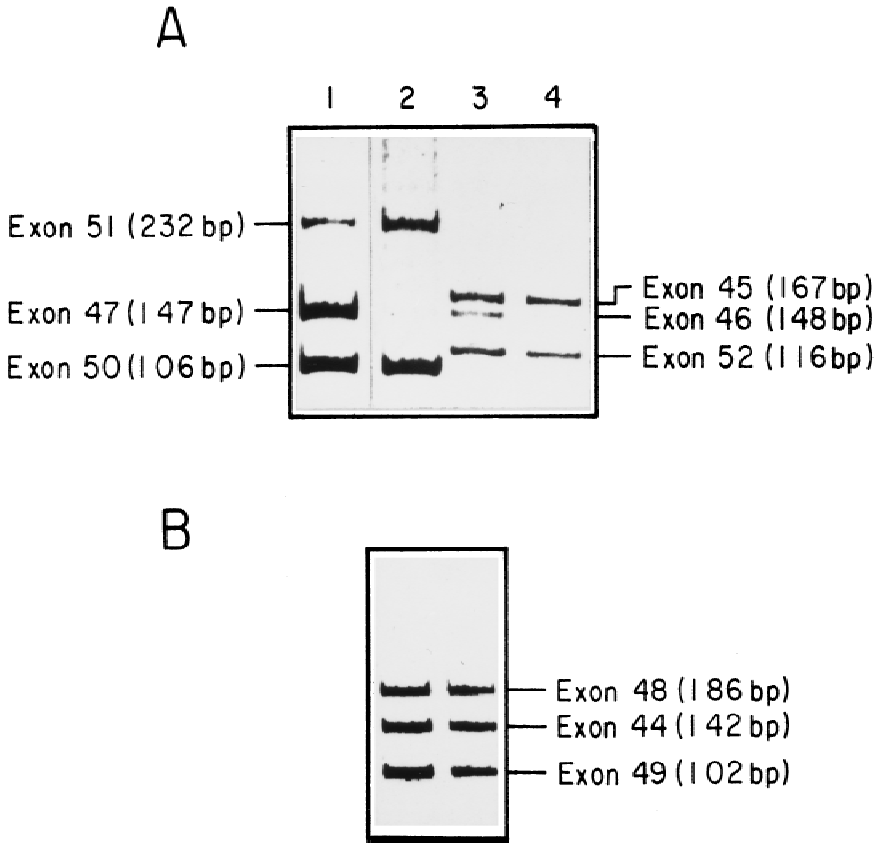

Fig. 3. 3-plex DNA amplification of genomic DNA from unrelated male DMD/BMD patients. Nine different regions of DMD gene were amplified, using three sets of 3-plex reactions, as described in Materials and Methods. PCR products were electrophoresed on 6-8% polyacrylamide gels stained with ethidium bromide. **A:** Representative gels displaying the DNA amplifications of exons 51/47/50 (**lanes 1** and **2**) and exons 45/46/52 (**lanes 3** and **4**). Lane 2 shows a deletion of exon 47. **B:** Representative gel displaying DNA amplification of exons 48/44/49 is shown. To the right of the panel the size of the amplified fragments in base pairs (bp) is indicated.

TABLE I. Frequencies and Distribution of Deletions in DMD/BMD Patients From Different Populations

	Cases	Deletion %	Breakpoints starting or terminating in		
			Major hot spot[a]	Minor hot spot[b]	Intron 44[c]
North America and Europe			%	%	%
Baumbach et al., 1989	160	56	69	29	24
Lindlof et al., 1989	90	50	58	27	11
Den Dunnen et al., 1989	194	59	63	18	31
Gillard et al., 1989	181	60	67	N.D.*	N.D.
Liechti-Gallati et al., 1989	95	66	68	22	24
Upadhyaya et al., 1990	164	50	68	18	32
Vitiello et al., 1992	115	56	86	9	40
Simard et al., 1992	77	68	63	23	25
Florentin et al., 1995	90	63	70	14	18
Asia					
Sugino et al., 1989	45	40	61	17	11
Asano et al., 1991	50	40	65	35	20
Zeng et al., 1991	46	56	73	11	N.D.
Kitoh et al., 1992	90	40	78	14	33
Imoto et al., 1993	88	61	63	13	17
Shomrat et al., 1994	62	37	78	20	14
Mexico					
Present study	59	53	87	6	26[c]

*N.D., not described.
[a]Deletions remove DNA fragments among exon 44 and exon 52.
[b]Deletions remove DNA fragments among exon 2 and exon 10.
[c]Between exons 44–45.

Most of our data 13/16 (81%) are in accordance with the translational reading frame hypothesis of Monaco et al. [1988]. We found exceptions to this rule in 2 DMD patients with in frame deletions of exons 45-51 and 48-51 and in one BMD patient with out of frame deletion of exon 44 (Fig. 2B). The analysis of the DMD cases showed that these deletions did not remove domains of a particular conformational importance [Passos-Bueno et al., 1994]. A possible explanation for the severe phenotype caused by the mutations includes early degradation of DMD mRNA [Chelly et al., 1990] or protein, effects on transcription, splicing and translation of the DMD gene. In the BMD case , the phenotype might be caused by alternative splicing creating an in-frame transcript [Chelly et al., 1990].

The prognosis for DMD/BMD patients based on the alteration of the reading frame is routinely carried out by the Southern blot technique using different cDNA probes of the DMD gene. The 3-plexs assays reported herein represent a less time consuming approach for such study; their use also overcomes several limitations of the Southern blot, namely, problems with comigrating fragments, weakly hybridizing fragments and identification of the exons in altered-size restriction fragments.

In this work we have described the deletion distribution in the DMD gene in Mexican patients. Based on the results presented, we consider that the use of the 3-plex assays described in this report represent a reliable alternative approach for a simpler and faster analysis of DMD/BMD Mexican patients.

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